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# Enhanced Therapeutic Efficacy of Poly(ICLC) and Ribavirin Combinations against Rift Valley Fever Virus Infection in Mice

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**The therapeutic efficacy of polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethyl cellulose [poly(ICLC)] given alone or in combination with ribavirin was evaluated in Swiss Webster mice infected with Rift Valley fever virus. Four or more 20-μg doses of poly(ICLC) given at various intervals beginning 24 h after infection protected all mice against death. On the other hand, a treatment regimen consisting of only three doses of poly(ICLC) given 24 h postinfection resulted in a 50% survival rate. When initiated 48 h postinfection, an extended treatment regimen with the same dose was required to yield 40% survivors. Lower doses (5 μg) of poly(ICLC) per mouse were only marginally effective even when six injections were given between days 1 and 9 postinfection. The combined administration of ribavirin and poly(ICLC) initiated as late as 48 h postinfection was effective even when treatment consisted of doses that were ineffective when either drug was used alone.**

Prophylactic treatments with polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine and carboxymethyl cellulose [poly(ICLC)] are effective in preventing a number of viral diseases in rhesus monkeys including simian hemorrhagic fever (11), rabies (1), yellow fever (17), Venezuelan equine encephalomyelitis (16), and Japanese encephalitis (5). In mouse models, poly(ICLC) is effective against rabies virus (1) and Venezuelan equine encephalomyelitis virus (9). In therapeutic studies, efficacy is retained when treatment is initiated as late as 8 and 24 h postinfection with yellow fever (17) and Japanese encephalitis (5) viruses, respectively. When treatment with poly(ICLC) is delayed 24 to 48 h after infection, the greater and more frequent doses required for effective treatment give rise to undesirable toxic side effects. High doses of ribavirin have also been used successfully to treat viral infections including experimental Rift Valley fever virus (RVFV) infections in several strains of mice (4). However, at low drug doses, treatment failures occur, resulting in death owing to either hepatitis or a subsequent encephalitis.

Poly(ICLC), as a potent interferon inducer, exerts its antiviral activity by interferon-mediated activation of nonspecific and specific immune responses including cytotoxic reactivity and helper cell functions (10). Interferon blocks mRNA capping by inhibiting transmethylation reactions (2, 8, 14), although this may be just one enzyme-related mechanism by which interferon can control viral proliferation (6). Ribavirin is also known to inhibit capping of mRNA by the inhibition of guanylyl transferase (14, 15).

In this report, we describe the therapeutic efficacy of poly(ICLC) against RVFV infection in mice. We also report the efficacy of low doses of poly(ICLC) given in combination with ribavirin. We emphasized the combinations of ribavirin and of low doses of poly(ICLC) which did not give rise to side effects and which were not effective when either compound was administered alone.

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## MATERIALS AND METHODS

**Antiviral compounds.** Poly(ICLC) in 0.9% sodium chloride solution was prepared by the Pharmaceutical Services, College of Pharmacy, University of Iowa (Iowa City). Each milliliter of poly(ICLC) solution contained 2 mg of poly(I)-poly(C), 1.5 mg of poly-L-lysine, and 5 mg of carboxymethyl cellulose. The pH was adjusted to 7.6 to 7.8 with sodium hydroxide. Ribavirin (1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide) was purchased from ICN Pharmaceuticals Inc. (Irving, Calif.). The drug was dissolved in sterile, injectable, pyrogen-free water. Both antiviral compounds were administered intraperitoneally (i.p.).

**Mice.** Female Swiss Webster mice (8 to 10 weeks old) were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Mice were housed for 5 to 14 days before they were tested.

**Virus.** The Zagazig Hospital 501 strain of RVFV was isolated during the 1977 epidemic in Cairo, Egypt. The virus was grown in cell culture, and titers were determined by a plaque assay (13). For quantitation, the virus was inoculated into 24-well culture plates containing 24-h-old, near-confluent Vero cells. Cultures were incubated at 37°C in 5% $CO_2$ for 60 min to allow adsorption of the virus before the addition of 0.5 ml of overlay medium (0.25% agarose in Eagle basal medium with Earle salt solution, supplemented with 16 mM HEPES [*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 7.5% heat-inactivated fetal bovine serum, and 5 μg of gentamicin per ml). Cells were incubated further at 37°C until plaques were visible. The tissue cultures were stained with 0.1% crystal violet for plaque counting. In in vivo efficacy studies, 250 PFU of virus per 0.1 ml was injected subcutaneously (s.c.).

**Statistics.** We calculated the statistical significance of therapeutic synergism by using the Cox model which defines the efficacy of the combination treatment in terms of incremental relative risk of death (7). The test computes whether the incremental risk of death of the combination treatment is significantly different from that obtained with a reference treatment with only one of the compounds. Further analysis

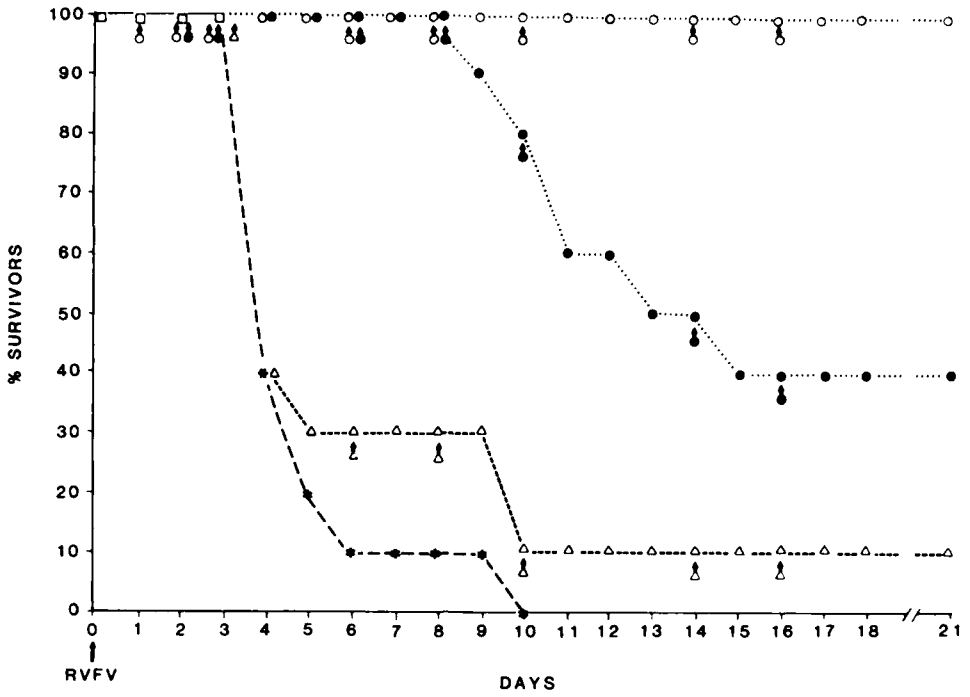


FIG. 1. Therapeutic efficacy of poly(ICLC) against RVFV infection in mice. Mice ($n$ = 10) were challenged s.c. on day 0 with 250 PFU of RVFV and injected i.p. with 20 μg of poly(ICLC) per mouse as follows: ○, days 1, 2, 3, 6, 8, 10, 14, 16; ●, days 2, 3, 6, 8, 10, 14, 16; △, days 3, 6, 8, 10, 14, 16; *, placebo. Days of treatment are indicated by arrows.

was made by using Fisher's exact test which considered only the total number of animals surviving in each treatment regimen.

## RESULTS

**Therapeutic efficacy of poly(ICLC).** We evaluated the efficacy of therapeutic schedules against RVFV infection of mice by using a six- to eight-dose regimen of poly(ICLC) (Fig. 1). We challenged Swiss Webster mice with 250 PFU of RVFV and then treated them with multiple 20-μg doses of poly(ICLC) beginning 24, 48, or 72 h postinfection. Additional doses were given at selected intervals through day 16. A majority of untreated mice died by day 5, and all untreated mice died by day 10. Treatment started 24, 48, or 72 h postinfection yielded survival rates of 100, 40, and 10%, respectively.

**Determining optimal dose and therapeutic treatment schedule.** Groups of Swiss Webster mice were challenged with 250 PFU of RVFV. Treatment was initiated 24 h postinfection with 20, 5, or 1 μg of poly(ICLC) per mouse. Experimental groups received a total of six, four, or three doses of poly(ICLC) at selected intervals through day 9 (Fig. 2). All mice given six or four injections of 20 μg of poly(ICLC) survived (Fig. 2A and B, respectively). However, a three-dose regimen of 20 μg of poly(ICLC) resulted in only a 50% survival rate (Fig. 2C). Regimens with poly(ICLC) doses of <20 μg were less effective even when six doses were administered (Fig. 2A). Treatment schedules consisting of three or four doses of 5 or 1 μg of poly(ICLC) were either marginally effective or not effective at all (Fig. 2B and C).

**Therapeutic efficacy of high doses of poly(ICLC) and ribavirin combinations.** To increase the rate of survival when treatment was initiated late in the course of disease, we designed a study using combinations of poly(ICLC) and ribavirin. For maximum efficacy, we administered six treatments between days 2 and 11 consisting of 20 μg of poly(ICLC) per mouse and 100 mg of ribavirin per kg of mouse (Fig. 3). The therapeutic efficacies of poly(ICLC) and ribavirin given individually were marginal, yielding 50 and 40% survivors, respectively, compared with 20% in placebo-treated controls. The combined therapy had an additive effect, increasing the survival rate to 80%. The survival rate obtained with the combination of high-dose poly(ICLC) and ribavirin was statistically highly significant ($P \leq 0.01$), when compared with that of the placebo-treated group. However, the increase was not significant ($P \geq 0.05$) in four separate experiments which compared combined treatment with treatment with either of the two drugs alone, although the results consistently showed increased survival rates (data not shown).

**Therapeutic effects of low doses of poly(ICLC) and ribavirin combinations.** We evaluated the therapeutic efficacy of combinations of poly(ICLC) and ribavirin regimens in treatment of RVFV-infected mice (Fig. 4). Challenge with 250 PFU of RVFV resulted in the death of 92% of untreated mice by day 6. Multiple treatments, beginning 24 h after challenge, with either 1 μg of poly(ICLC) or 50-, 25-, or 12.5-mg/kg ribavirin alone resulted in 17% survival for poly(ICLC) and 33, 8, and 0% long-term survivors, respectively, for ribavirin. However, a greater efficacy was obtained when 1 μg of poly(ICLC) was combined with 50-, 25-, or 12.5-mg/kg ribavirin to yield 75, 92, and 58% survival rates, respectively.

Ranking of treatment efficacies by their incremental relative risk of death (Cox model) indicated that ribavirin

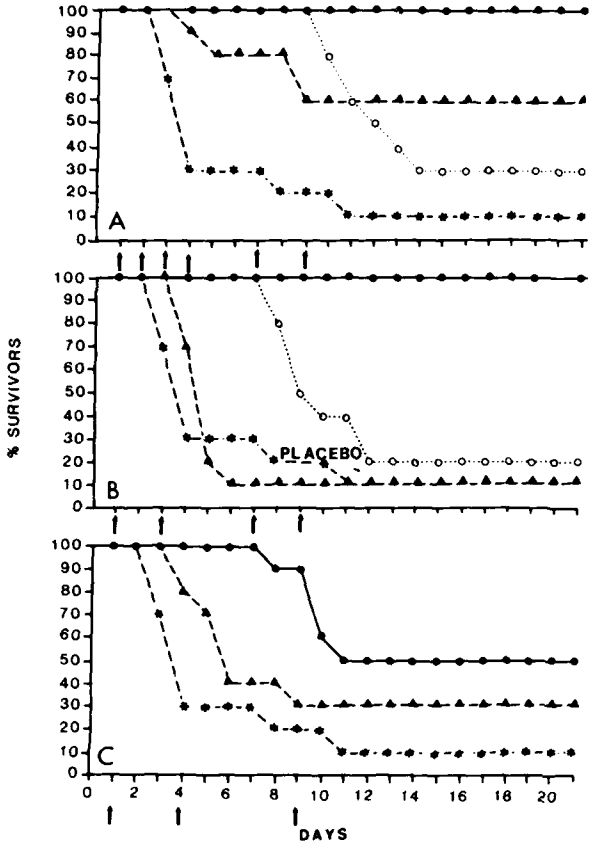


FIG. 2. Determination of optimal therapeutic regimen for poly(ICLC) against RVFV infection in mice. Mice ($n$ = 10) were challenged s.c. on day 0 with 250 PFU of RVFV and injected i.p. with poly(ICLC) as follows: ●, 20 μg per mouse; ○, 5 μg per mouse; ▲, 1 μg per mouse; *, placebo. Days of treatment are indicated by the arrows.

therapy alone at all three doses was comparable to the reference standard therapy of poly(ICLC) because the respective incremental relative risks of death were not significantly different (Table 1). On the other hand, combination treatment regimens with poly(ICLC) and three levels of ribavirin resulted in incremental relative risks of death which were significantly lower ($P < 0.01$) compared with those of the standard reference poly(ICLC) regimen. Based on total survivors at the end of the experiment, the combination therapy was highly significant (Fisher's exact test) when 25- and 50-mg/kg ribavirin was administered ($P < 0.01$), but it was only marginally significant with 12.5-mg/kg ribavirin ($P < 0.05$).

In a second study, treatment with 5 μg of poly(ICLC) or 50-, 25-, or 12.5-mg/kg ribavirin alone or in combination was initiated 48 h after challenge with 250 PFU or RVFV. All but one placebo-treated mouse died by day 5, and treatment with either poly(ICLC) or ribavirin alone was not efficacious. However, treatment with 5 μg of poly(ICLC) in combination with 50-, 25-, or 12.5-mg/kg ribavirin yielded long-term survivors (Fig. 5). Ranking the efficacy of various treatment regimens on the basis of incremental relative risk of death indicated that the 25- and 50-mg/kg ribavirin regimens were as efficacious as the reference regimen of 5 μg of poly(ICLC) (Table 2) but that the 12.5-mg/kg dose regimen was less effective. The results of the combination of 5 μg of poly(ICLC) and 12.5-mg/kg ribavirin were indistinguishable

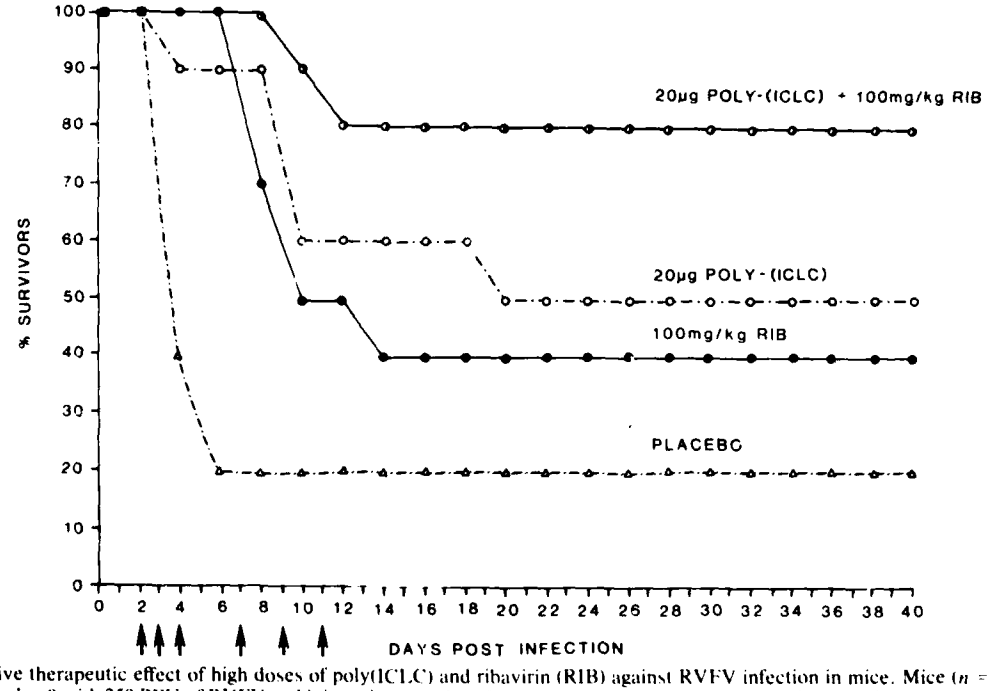


FIG. 3. Additive therapeutic effect of high doses of poly(ICLC) and ribavirin (RIB) against RVFV infection in mice. Mice ($n$ = 10) were challenged s.c. on day 0 with 250 PFU of RVFV and injected i.p. on days 2, 3, 4, 7, 9, and 11 postinfection as follows: ◑, 20 μg of poly(ICLC) per mouse plus 100 mg of ribavirin per kg; ○, 20 μg of poly(ICLC) per mouse; ●, 100 mg of ribavirin per kg; △, placebo. Days of treatment are indicated by arrows.

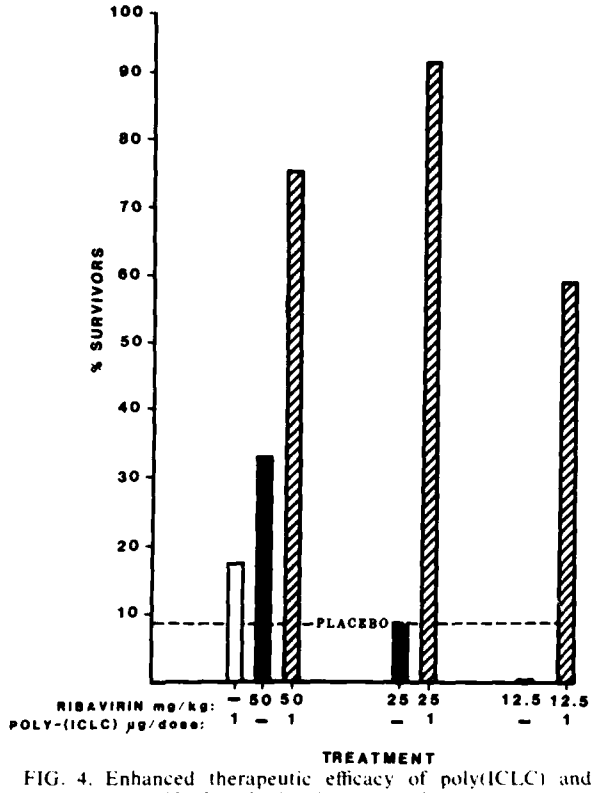


FIG. 4. Enhanced therapeutic efficacy of poly(ICLC) and ribavirin against RVFV infection in mice. Mice ($n$ = 12) were challenged s.c. with 250 PFU of RVFV on day 0 and injected i.p. 24 h postinfection as follows: □, 1 μg of poly(ICLC) per mouse on days 1, 4, and 9; ■, 50, 25, or 12.5 mg of ribavirin per kg on days 1 to 4, 7, 9, and 11; ▨, 1 μg of poly(ICLC) per mouse on days 1, 4, and 9 plus 50, 25, or 12.5 mg of ribavirin per kg on days 1 to 4, 7, 9, 11; dashed line, placebo.

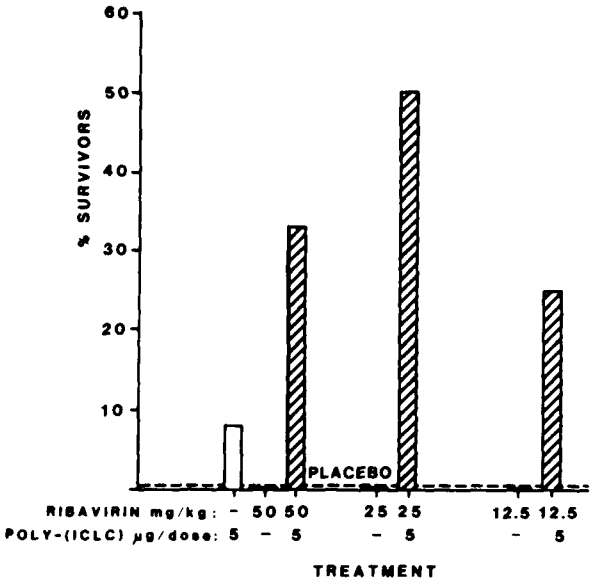


FIG. 5. Enhanced therapeutic efficacy of poly(ICLC) and ribavirin against RVFV infection in mice. Mice ($n$ = 12) were challenged s.c. with 250 PFU of RVFV on day 0 and injected i.p. 48 h postinfection as follows: □, 5 μg of poly(ICLC) per mouse on days 2, 3, 4, 6, and 8; ■, 50, 25, or 12.5 mg of ribavirin per kg on days 2, 3, 4, 6, 8, and 10; ▨, 5 μg of poly(ICLC) on days 2, 3, 4, 6, and 8 plus 50, 25, or 12.5 mg of ribavirin per kg on days 2, 3, 4, 6, 8, and 10; dashed line, placebo.

from the results of the standard treatment. According to the Cox model, the combination of poly(ICLC) and 25- or 50-mg/kg ribavirin ranked significantly higher than poly(ICLC) alone ($P$ < 0.05), indicating a trend (albeit a decreased one) even when the initiation of treatment was delayed. However, the combination therapy was not signif-

TABLE 1. Efficacy ranking of combination therapy with poly(ICLC) and ribavirin initiated 24 h postinfection in RVFV infected mice

| Treatment regimen | No. of survivors/total | Incremental relative risk of death[a] | *P* value | |
|---|---|---|---|---|
| | | | Cox | Fisher[b] |
| 12.5-mg/kg ribavirin | 0/12 | 1.75 | 0.2014[c] | 0.2391[c] |
| 1 μg of poly(ICLC) (standard treatment) | 2/12 | 1.00 | | |
| 25-mg/kg ribavirin | 1/12 | 0.68 | 0.3842[c] | 0.5000[c] |
| 50-mg/kg ribavirin | 4/12 | 0.46 | 0.1060[c] | 0.3202[c] |
| 1 μg of poly(ICLC) + 12.5-mg/kg ribavirin | 7/12 | 0.22 | 0.0065[d] | 0.0447[d] |
| 1 μg of poly(ICLC) + 50-mg/kg ribavirin | 8/12 | 0.10 | 0.0014[d] | 0.0061[d] |
| 1 μg of poly(ICLC) + 25-mg/kg ribavirin | 11/12 | 0.03 | 0.0007[d] | 0.0003[d] |

[a] Efficacy ranking of combination therapies based on incremental relative risk of death against a standard treatment.
[b] One-tail exact test.
[c] $P$ > 0.05; indistinguishable from standard treatment.
[d] $P$ < 0.01; significantly fewer animals at risk.

TABLE 2. Efficacy ranking of combination therapy with poly(ICLC) and ribavirin initiated 48 h postinfection in RVFV infected mice

| Treatment regimen | No. of survivors/total | Incremental relative risk of death[a] | *P* value | |
|---|---|---|---|---|
| | | | Cox | Fisher[b] |
| 12.5-mg/kg ribavirin | 0/12 | 5.14 | 0.0006[d] | 0.5000[c] |
| 25-mg/kg ribavirin | 0/12 | 1.64 | 0.2419[c] | 0.5000[c] |
| 50-mg/kg ribavirin | 0/12 | 1.21 | 0.6429[c] | 0.5000[c] |
| 5 μg of poly(ICLC) (standard treatment) | 1/12 | 1.00 | | |
| 5 μg of poly(ICLC) + 12.5-mg/kg ribavirin | 3/12 | 0.48 | 0.1123[c] | 0.2950[c] |
| 5 μg of poly(ICLC) + 50-mg/kg ribavirin | 4/12 | 0.39 | 0.0498[d] | 0.1584[c] |
| 5 μg of poly(ICLC) + 25-mg/kg ribavirin | 5/12 | 0.34 | 0.0006[d] | 0.0775[c] |

[a] Efficacy ranking of combination therapies based on incremental relative risk of death against a standard treatment.
[b] One-tail exact test.
[c] $P$ > 0.05; significantly more animals at risk than with standard treatment.
[d] $P$ > 0.05; indistinguishable from standard treatment.
[e] $P$ < 0.05; significantly fewer animals at risk.

icantly better than the standard treatment when examined with Fisher's exact test.

## DISCUSSION

RVFV infection is uniformly lethal to mice within 4 to 6 days of challenge. When treatment was initiated within 24 h after infection, complete protection was obtained with a regimen consisting of four or more doses of 20 μg of poly(ICLC). Even when treatment was delayed for 48 h, a 40 to 50% survival rate was obtained with the same dose of poly(ICLC). An equivalent therapeutic dose of poly(ICLC) for humans, however, is known to produce severe toxicity (12), which argues against the use of such high doses of this drug. Multiple high doses of poly(ICLC) and ribavirin in combination resulted in an additive effect in efficacy. Such combinations were effective even when treatment was initiated 48 h postinfection. Although the additive effect was not statistically significant with respect to either ribavirin or poly(ICLC) therapy alone, such additive effects were observed consistently in all the therapeutic combinations employing high doses of these antiviral compounds. Similar additive effects were reported with 150 mg of ribavirin per kg and 20 μg of poly(G)-poly(C) against tick-borne encephalitis virus infection of mice (18).

Enhanced therapeutic effects (as high as 92% protection) became quite evident when lower doses of poly(ICLC) and ribavirin were administered in combination beginning 24 h postinfection. Enhancement of the efficacy was highly significant by the Cox model and Fisher's exact test. This was in sharp contrast to the lack of protection when similar doses of either compound were used alone. This trend was considerably diminished when the treatment was delayed until 48 h, necessitating a fivefold increase in poly(ICLC) and higher doses of ribavirin to obtain a 50% protection rate. With a 48 h delay, enhancement of the efficacy was significant when examined with the Cox model, but not with Fisher's exact test. Postinfection therapy with low doses of ribavirin or poly(ICLC) alone failed to protect mice effectively. However, enhanced therapeutic activity can be achieved by the use of the two drugs in combination, presumably by inhibiting viral proliferation by simultaneous blocking of different capping events (2, 8, 14, 15). Enhancement of the therapeutic efficacy by combination therapy with compounds having different modes of action offers an attractive therapeutic approach for treatment of human disease. Adverse side effects would not be expected during a 7- to 14-day therapy with small doses of poly(ICLC) and ribavirin. Even high doses of ribavirin are well tolerated in humans (3).

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